EZH2 and the polycomb repressor complex 2 are required for the reprogramming of Müller Glia into progenitor cells in the retina.

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Abstract:

Purpose: Many retinal diseases lead to the loss of retinal neurons, resulting in significant visual impairment and blindness. In mammals, there is no regenerative response to replenish lost neurons and restore vision. However, retinal regeneration occurs in chick and zebrafish where lost neurons are regenerated. Müller glia, the primary glial cell of the retina, are the source of these regenerated neurons. Müller glia detect retinal damage, de-differentiate into progenitor cells, and progeny differentiate to become neurons. The transition from glia to neuron requires reorganization of chromatin architecture, which is accomplished through epigenetic regulation and histone modifications. The polycomb repressor complex 2 (PRC2) is a histone methylase that regulates retinal development, and knockout of the catalytic subunit EZH2 results in microphthalmia. Thus, we hypothesize that the PRC2 complex is necessary for the repression of glial fate genes, permitting Müller glia to de-differentiation and become progenitor-like cells. Therefore, we investigated the epigenetic regulation of Müller glia reprogramming.

Research Methods: Postnatal White Leghorn chicks (P7-14) were intravitreally injected with excitotoxin N-methyl-D-aspartate (NMDA) to induce retinal damage and initiate Müller glia reprogramming. We administered EZH2 inhibitor 3-Deazaneplanocin A (DZN) to determine the impact on the formation of proliferating Müller glia derived progenitor cells. We enucleated, fixed,

and sectioned the tissue for fluorescence immunohistochemistry (IHC). We quantified the number of progenitors that entered the cell cycle by staining for the incorporation of nuclear 5-Ethynyl-2'-deoxyuridine (EdU) to colocalize with Müller glia/progenitor markers Sox2/Sox9. Retinal preparations were dissociated for single cell analysis. We performed single cell RNA sequencing (scRNA-seq) and a single cell Assay for Transposase-Accessible Chromatin sequencing (scATAC-seq) on damaged, undamaged, and DZN treated retinas to determine how changes in chromatin remodeling and gene expression occurred during reprogramming and were influenced by inhibition of EZH2.

Results: We found that *EZH2* and other components of the PRC2 complex are specifically upregulated in Müller glia during the de-differentiation process to form Müller glia derived progenitor cells. Single cell sequencing indicates an upregulation *of EZH2, EED, SET* and *RBBP4*. IHC of EZH2 protein also colocalized with Müller glia nuclei after damage. The inhibition of EZH2 with DZN blocked the formation of progenitor cells after damage. scRNA-seq analysis demonstrated that EZH2-inhibition significantly influenced gene expression and chromatin access. This led to increased expression in glial transcription factors (*ID4, NFIA, SOX8*) in damaged retinas, while also increasing reactive glial genes (*ATF4, NFIX, NEFM, DCX, HES1*). When observing chromatin accessibility in scATAC-seq data, DZN treatment increased access to many important genes, including pro-glial factors such as *ID4* and *NFIX*. Genomic regions surrounding both glial (*NOTCH1, GLI2*) and neuronal (*HEYL1, LHX9*) transcription factors were also more accessible as a result of inhibiting the PRC2 complex.

Implications: We find that PRC2 complex genes are upregulated in Müller glia-derived progenitors during reprogramming. We demonstrate the pharmacological inhibition of EZH2 robustly negates the transition from glia to progenitor cell after damage. Single cell analyses of

chromatin accessibility indicate that this repressive histone methylase condenses chromatin around transcription factors that promote mature glial or neuronal phenotype. These epigenetic modifications correlate to the increased expression glial genes the repress a progenitor cell phenotype. We identify genes that may be key regulators of Müller glia's response to damage and are required for the transition into progenitor cells. These findings provide new insight into how epigenetic factors impact regeneration in the retina and central nervous system. Future experimentation is required to study how histone modifications influence the poor regenerative response in the retinas of mammals.

Introduction:

Müller glia (MG) are the predominant type of support cell in the retina. These glia are common to all vertebrate classes. MG perform many important glial functions including structural support, synaptic support, osmotic homeostasis, and nutritive/metabolic support to retinal neurons. However, MG also have the extraordinary capability to de-differentiate, proliferate, acquire progenitor phenotype, and generate new neurons (1–5). It must be noted that in normal, healthy retinas MG are distinctly different from retinal progenitor cells based on cellular functions and transcriptional profile. It is believed that cellular reprogramming underlies that the transition of MG into progenitor cells. Reprogramming of MG involves down-regulation of glial genes, up-regulation of progenitor-associated factors, proliferation, followed by neuronal differentiation. Cellular proliferation is thought to be an integral step of MG becoming progenitor-like cells (6,7). In the chick model system, we were the first to demonstrate that MG can become progenitor cells that regenerate neurons (8). In response to N-methyl-D-aspartate (NMDA) -induced damage, numerous MG de-differentiate, re-enter the cell-cycle, and express transcription factors which are found in embryonic retinal progenitors (8–11). In response to sufficient levels of neuronal damage, numerous MG undergo a single round of division *in vivo*; these cells continue to proliferate when they are dissociated and grown in culture (8), suggesting that the intact retina provides cues to suppress the formation of Müller glia-derived progenitor cells (MGPCs). *In vivo*, the majority (about 80%) of cells that are generated by proliferating MGPCs remain as un-differentiated progenitor cells, some differentiate into MG and a few differentiate into neurons. Although few neurons are regenerated, MGPCs produce thousands of undifferentiated cells that could be stimulated to differentiate as neurons to restore vision.

With a few exceptions, the formation of MGPCs is stimulated by severe acute retinal damage. One exception is consecutive daily injections of Fibroblast Growth Factor 2 (FGF2) and/or insulin/IGF1 which activates a network of cell-signaling pathways to stimulate the formation of neurogenic MGPCs in the chick retina, in the absence of damage (12,13), but this requires the presence of reactive microglia (13). In the zebrafish retina, activation of Wnt-signaling or MAPK-signaling via HB-EGF or FGF+insulin can stimulate the formation of MGPCs and *de novo* neurogenesis (14–16). Further, TNFα combined with Notch-inhibitor is sufficient to stimulate the formation of MGPCs and *de novo* neurogenesis in zebrafish retina (17,18). In the mouse model, recent studies indicated that forced-expression of the transcription factor *Ascl1a*, in combination with NMDA-induced damage and HDAC-inhibitor, stimulates reprogramming of MG into functional, light-responsive neurons (19–21). Further evidence in the mouse suggests that activation of Wnt/β-catenin and forced expression of photoreceptor transcription factors (*Otx2, Crx, Nrl*) stimulates proliferation and reprograming of MG into rod photoreceptors (22). It makes no sense to stimulate neural regeneration from MG in slowly degenerating retinas by inducing severe, acute damage. Thus, identification of the secreted factors, transcription factors and signaling pathways that stimulate MG without further damaging retinal neurons is crucial to harnessing the neurogenic potential of these glia.

Our findings in the chick retina have guided studies involving MG as a cellular source of neural regeneration in mammals (4,5) and zebrafish (3,23). By comparison, MG in the fish retina have an extraordinary capacity to regenerate retinal neurons whereas MG in the mammalian retina have very restricted capacity. Interestingly, MG in the chick retina have a neurogenic potential that lies between than of fish and mammals. Collectively, these observations raise

important questions as to why MG in fish regenerate numerous functional neurons, whereas this potential is restricted in birds and blocked in mammals?

The purpose of the studies proposed herein is to understand how the activity of EZH2 regulates the dedifferentiation of MG, reprogramming into proliferating MGPCs, and the neuronal differentiation of the progeny of MGPCs. There is nothing known regarding the roles of EZH2 (enhancer of zeste 2) and polycomb repressor complex (PRC2) in mature MG and the formation of MGPCs in the retina. EZH2 is histone-lysine N-methyltransferase that can influence many cellular processes including proliferation and differentiation. The canonical functions of EZH2 involve participation in the polycomb repressive complex 2 (PRC2) which suppresses transcription via tri-methylation of H3K27. PRC2 is comprised not only EZH2, but also regulatory/catalytic subunits EED, SUZ12, RBP48 and SET. EZH2 is also known to have non-canonical functions which can include methylation of Stat3 and RORα, transcriptional activation following phosphorylation by JAK3, and methylation-independent promotion of cyclin D, Notch1 and Wnt-related genes (24,25). EZH2 is known to promote the proliferation of early stage retinal progenitors and differentiation of late-born neurons and glia (26–28). Further, embryonic deletion of *Ezh2* from the retina results in postnatal degeneration of photoreceptors, likely because of failure to suppress embryonic transcription factors (29). Some of the gene-repressing actions of EZH2 may be guided by interactions Six3 and Six6, as well as the lcRNA Six3OS (29,30), and these genes are prominently expressed by MG and MGPCs in normal and damaged retinas according to preliminary data from scRNA-seq on cells from mouse and chick retinas (not shown). Currently, there is nothing known about the functions of EZH2 in mature MG and during the formation of MGPCs. Accordingly, we propose to investigate how EZH2 influences glial phenotype, de-differentiation MG, proliferation of MGPCs and neuronal

differentiation *in vivo*. My laboratory will investigate how EZH2-activity impacts MG and MGPCs using the chick and mouse model systems. The chick model provides an efficient and cost-effective means to investigate the efficacy and specificity of pharmacological agents upon MG and MGPCs. The eyes of chicks are large and readily tolerate repeated intraocular injections. In future studies, we will compare data from zebrafish, chick and rodent model systems, which is expected to provide important, novel insights into the differences in MG plasticity and regenerative capacity of MGPCs. For example, a comparative cross-species approach has been applied to generate significant volumes of data using RNA-seq, scRNA-seq and ATAC-seq of MG and MGPCs from zebrafish, chick and mouse retinas (31). There already exists significant volumes of data regarding the networks of cell-signaling pathways and transcription factors that are involved in the formation of MGPCs in different the different model systems (Reviewed by (32,33)).

Methods and Materials:

Animals:

The animals approved for use in these experiments was in accordance with the guidelines established by the National Institutes of Health and IACUC at The Ohio State University. Newly hatched P0 wildtype leghorn chicks (*Gallus gallus domesticus*) were obtained from Meyer Hatchery (Polk, Ohio). Post-hatch chicks were maintained in a regular diurnal cycle of 12 hours light, 12 hours dark (8:00 AM-8:00 PM). Chicks were housed in stainless-steel brooders at 25^{o}C and received water and Purina^{tm} chick starter *ad libitum*.

Fertilized eggs were obtained from the Michigan State University, Department of Animal Science. Eggs were incubated at a constant 37.5°C, with a 1hr period room temperature cool down every 24hrs. Additionally, the eggs were rocked every 45 minutes, and held at a constant relative humidity of 45%. Embryos were harvested at various time points after incubation and staged according to guidelines established by Hamburger and Hamilton (1951).

Intraocular injections:

Chicks were anesthetized with 2.5% isoflurane mixed with oxygen from a non-rebreathing vaporizer. The technical procedures for intraocular injections were performed as previously described (Fischer et al., 1998). With all injection paradigms, both pharmacological and vehicle treatments were administered to the right and left eye respectively. Compounds were injected in 20 ml sterile saline with 0.05 mg/ml bovine serum albumin added as a carrier. Compounds included: NMDA (38.5nmol or 154 µg/dose; Sigma-Aldrich), FGF2 (250 ng/dose; R&D systems), DZNexp4 (200 ng/dose; Tocris), EEDi (200 ng/dose; Tocris), UNC1999 (200 ng/dose; Tocris), GSK126 (200 ng/dose; Tocris), GSK343 (200 ng/dose; Tocris), JIB 04 (200

ng/dose; Tocris). 5-Ethynyl-2´-deoxyuridine (EdU) was intravitreally injected to label the nuclei of proliferating cells. Injection paradigms are included in each figure.

Preparation of clodronate liposomes:

Clodronate liposomes were synthesized utilizing a modified protocol from previous descriptions (Van Rooijen, 1989; van Rooijen, 1992; Zelinka et al., 2012). In short, approximately 8 mg of L-α-Phosphatidyl-DL-glycerol sodium salt (Sigma P8318) was dissolved in chloroform. 50 mg of cholesterol was dissolved in chloroform with the lipids in a sterile microcentrifuge tube. This tube was rotated under nitrogen gas to evaporate the chloroform and leave a thin lipid-film on the walls of the tube. 158 mg dichloro-methylene diphosphonate (clodronate; Sigma-Aldrich) dissolved sterile PBS (pH 7.4) was added to the lipid/cholesterol film and vortexed for 5 minutes. To reduce size variability of lipid vesicles, the mixture was sonicated at 42 kHz for 6 minutes. Purification of liposomes was accomplished via centrifugation at 10,000 x G for 15 minutes, aspirated, and resuspended in 150 µl PBS. Each retinal injection used between 5 and 20 ul of clodronate-liposome solution. There was a variable yield of clodronate-liposomes during the purification resulting in some variability per dose. The dosage was adjusted such that >98% of the microglia are ablated by 2 days after administration with no off-target cell death or pigmented epithelial cells.

Single Cell RNA and ATAC sequencing of retinas

Retinas were obtained from embryonic and postnatal chicks. Isolated retinas were dissociated in a 0.25% papain solution in Hank's balanced salt solution (HBSS), pH = 7.4, for 30 minutes, and suspensions were frequently triturated. The dissociated cells were passed through a

sterile 70µm filter to remove large particulate debris. Dissociated cells were assessed for viability (Countess II; Invitrogen) and cell-density diluted to 700 cell/µl. Each single cell cDNA library was prepared for a target of 10,000 cells per sample. The cell suspension and Chromium Single Cell 3' V2 reagents (10X Genomics) were loaded onto chips to capture individual cells with individual gel beads in emulsion (GEMs) using 10X Chromium Controller. cDNA and library amplification for an optimal signal was 12 and 10 cycles respectively. Sequencing was conducted on Illumina HiSeq2500 (Genomics Resource Core Facility, John's Hopkins University) or HiSeq4000 (Novogene) with 26 bp for Read 1 and 98 bp for Read 2. Fasta sequence files were de-multiplexed, aligned, and annotated using the chick ENSMBL database (Chick-5.0, Ensembl release 94) and Cell Ranger software. Gene expression was counted using unique molecular identifier bar codes, and gene-cell matrices were constructed. Using Seurat toolkits, t-distributed stochastic neighbor embedding (tSNE) plots or Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) plots were generated from aggregates of multiple scRNA-seq libraries (Butler et al., 2018; Satija et al., 2015). Compiled in each tSNE/UMAP plot are two biological library replicates for each experimental condition. Seurat was used to construct violin/scatter plots. Significance of difference in violin/scatter plots was determined using a Wilcoxon Rank Sum test with Bonferroni correction. Monocle was used to construct unbiased pseudo-time trajectories and scatter plotters for MG and MGPCs across pseudotime (Qiu et al., 2017a; Qiu et al., 2017b; Trapnell et al., 2012). Genes that were used to identify different types of retinal cells included the following: (1) Müller glia: *GLUL, VIM, SCL1A3, RLBP1*, (2) MGPCs: *PCNA, CDK1, TOP2A, ASCL1*, (3) microglia: *C1QA, C1QB, CCL4, CSF1R, TMEM22*, (4) ganglion cells: *THY1, POU4F2, RBPMS2, NEFL, NEFM*, (5) amacrine cells: *GAD67, CALB2, TFAP2A*,

(6) horizontal cells: *PROX1, CALB2, NTRK1,* (7) bipolar cells: *VSX1, OTX2, GRIK1, GABRA1*, and (7) cone photoreceptors: *CALB1, GNAT2, OPN1LW,* and (8) rod photoreceptors: *RHO, NR2E3, ARR3.* The MG have an over-abundant representation in the scRNA-seq databases. This likely resulted from fortuitous capture-bias and/or tolerance of the MG to the dissociation process. scRNA-seq libraries for retinas treated with NMDA and/or insulin and FGF2 can be queried at: https://proteinpaint.stjude.org/F/2019.retina.scRNA.html

Fixation, sectioning and immunocytochemistry:

Retinal tissue samples were formaldehyde fixed, sectioned, and labeled via immunohistochemistry as described previously (Fischer et al., 2008; Fischer et al., 2009c). Antibody dilutions and commercial sources for images used in this study are described in table 2. Observed labeling was not due to off-target labeling of secondary antibodies or tissue autofluorescence because sections incubated exclusively with secondary antibodies were devoid of fluorescence. Secondary antibodies utilized include donkey-anti-goat-Alexa488/568, goat-anti-rabbit-Alexa488/568/647, goat-anti-mouse-Alexa488/568/647, goat-anti-rat-Alexa488 (Life Technologies) diluted to 1:1000 in PBS and 0.2% Triton X-100.

Labeling for EdU:

For the detection of nuclei that incorporated EdU, immunolabeled sections were fixed in 4% formaldehyde in 0.1M PBS pH 7.4 for 5 minutes at room temperature. Samples were washed for 5 minutes with PBS, permeabilized with 0.5% Triton X-100 in PBS for 1 minute at room temperature and washed twice for 5 minutes in PBS. Sections were incubated for 30 minutes at room temperature in a buffer consisting of 100 mM Tris, 8 mM $CuSO_4$, and 100 mM ascorbic

acid in dH_2O. The Alexa Fluor 568 Azide (Thermo Fisher Scientific) was added to the buffer at a 1:100 dilution.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL):

The TUNEL assay was implemented to identify dying cells by imaging fluorescent labeling of double stranded DNA breaks in nuclei. The *In Situ* Cell Death Kit (TMR red; Roche Applied Science) was applied to fixed retinal sections as per the manufacturer's instructions.

Photography, measurements, cell counts and statistics:

Microscopy images of retinal sections were captured with the Leica DM5000B microscope with epifluorescence and the Leica DC500 digital camera. High resolution confocal images were obtained with a Leica SP8 available in The Department of Neuroscience Imaging Facility at The Ohio State University. Representative images are modified to have enhanced color, brightness, and contrast for improved clarity using Adobe Photoshop. For quantification of numbers of EdU^+ cells, a fixed region of retina was counted and average numbers of Sox2+/CD45+ and EdU co-labeled cells. The retinal region selected cell counts was standardized between treatment and control groups to reduce variability and improve reproducibility.

Similar to previous reports (Fischer et al., 2009a; Fischer et al., 2009b; Ghai et al., 2009), immunofluorescence was quantified by using ImagePro6.2 (Media Cybernetics, Bethesda, MD, USA) or Image J (NIH). Identical illumination, microscope, and camera settings were used to obtain images for quantification. Retinal areas were sampled from 5.4 MP digital images. These areas were randomly sampled over the inner nuclear layer (INL) where the nuclei of the bipolar and amacrine neurons were observed. The density sum was calculated as the total of pixel values

for all pixels within thresholded regions. The mean density sum was calculated for the pixels within threshold regions from ≥5 retinas for each experimental condition. GraphPad Prism 6 was used for statistical analyses.

For statistical evaluation of differences in treatments, a two-tailed paired *t*-test was applied for intra-individual variability where each biological sample also served as its own control. For two treatment groups comparing inter-individual variability, a two-tailed unpaired *t*-test was applied. For multivariate analysis, an ANOVA with the associated Tukey Test was used to evaluate any significant differences between multiple groups.

Results & Conclusions

The role of EZH2 was investigated in the formation of Müller glia progenitor cells after NMDA damage. First, using single cell RNA sequencing, we were able to probe the different cell types of the chick retina to detect the various different components of the PRC2 complex, such as the catalytic unit EZH2. After damage, the forming progenitor cells upregulate EZH2, along with other cell cycle markers (Fig1a-e). This upregulation can also be visualized through the violin plot and pseudotime which models the transition from glial cell to progenitor cell Fig(1e-g).

The upregulation of the transcripts of EZH2 was also matched with a increase in the protein expression of EZH2 (Fig.2). EZH2 is not detected in Müller glia in undamaged retina, but after 48hrs, the nuclei of Sox9 positive Müller glia were positive for EZH2 immunolabelling (Fig2a-c). Other cells were positive for EZH2 as well, including microglia as evidenced by the CD45 EZH2 colocalization (Fig2c). This suggests that EZH2 has functional roles in cellular proliferation in addition to potential roles facilitating the reprogramming of Müller glia.

To establish a functional dependence on EZH2 function for reprogramming, we used DZN as a target specific small molecule inhibitor after damage and measure the number of proliferating progenitor cells. We observed that with the administration of DZN, there was a significant decrease in the number EdU^+ $Sox2^+$ progenitor cells in the chick retina (Fig 3). This shows that there is a functional dependence on the function of EZH2 to enable the process of de-differentiation and proliferation of Müller glia into progenitor like cells.

We took the DZN inhibited retinas, and created scRNA-seq libraries of the retinas with and without NMDA excitotoxic damaged retinas. The results showed that the DZN treated retinas without damage were significantly different than the DZN untreated retinas, as they expressed several more glia markers and transcription factors while also downregulating the amount of proliferation factors (Fig 4). Similarly, this was also true of the NMDA plus DZN treated retinas, but to a lesser degree. We are still investigating why there was such a larger significant difference in the undamaged condition. Part of the limitation of the NMDA DZN treated retinas is that there was a very limited sample size of MG that passed our quality control metrics, thus limited the amount of analysis that could be done.

In conclusion, this data provides novel evidence that the PRC2 complex, specifically the catalytic unit EZH2 serves a necessary role in transitioning mature Müller glia into proliferating progenitor cells. The large data collected from the single cell RNA sequencing suggests that EZH2 may normally be repressing pro-glial genes that repress the de-differentiation process and that without this epigenetic repression, the regenerative capacity of these cells between species may be limited. Future work will be validating the regions of epigenetic modulation to improve the efficiency of this cellular conversion.

Citations

1. Fausett BV, Gumerson JD, Goldman D. The proneural basic helix-loop-helix gene ascl1a is required for retina regeneration. J Neurosci. 2008 Jan 30;28(5):1109–17.

2. Fischer AJ, Reh TA. Muller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat Neurosci. 2001 Mar;4(3):247–52.

3. Bernardos RL, Barthel LK, Meyers JR, Raymond PA. Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J Neurosci. 2007 Jun 27;27(26):7028–40.

4. Ooto S, Akagi T, Kageyama R, Akita J, Mandai M, Honda Y, et al. Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. Proc Natl Acad Sci U A. 2004 Sep 14;101(37):13654–9.

5. Karl MO, Hayes S, Nelson BR, Tan K, Buckingham B, Reh TA. Stimulation of neural regeneration in the mouse retina. Proc Natl Acad Sci U A. 2008 Dec 9;105(49):19508–13.

6. Fischer AJ, Bongini R. Turning Müller glia into neural progenitors in the retina. Mol Neurobiol. 2010 Dec;42(3):199–209.

7. Fischer AJ, Reh TA. Potential of Müller glia to become neurogenic retinal progenitor cells. Glia. 2003 Jul;43(1):70–6.

8. Fischer AJ, Reh TA. Müller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat Neurosci. 2001 Mar;4(3):247–52.

9. Fischer AJ, Scott MA, Ritchey ER, Sherwood P. Mitogen-activated protein kinase-signaling regulates the ability of Müller glia to proliferate and protect retinal neurons against excitotoxicity. Glia. 2009 Nov 1;57(14):1538–52.

10. Fischer AJ, Scott MA, Tuten W. Mitogen-activated protein kinase-signaling stimulates Müller glia to proliferate in acutely damaged chicken retina. Glia. 2009 Jan 15;57(2):166–81.

11. Todd L, Fischer AJ. Hedgehog signaling stimulates the formation of proliferating Müller glia-derived progenitor cells in the chick retina. Dev Camb Engl. 2015 Aug 1;142(15):2610–22.

12. Fischer AJ, McGuire CR, Dierks BD, Reh TA. Insulin and fibroblast growth factor 2 activate a neurogenic program in Müller glia of the chicken retina. J Neurosci Off J Soc Neurosci. 2002 Nov 1;22(21):9387–98.

13. Fischer AJ, Zelinka C, Gallina D, Scott MA, Todd L. Reactive microglia and macrophage facilitate the formation of Müller glia-derived retinal progenitors. Glia. 2014 Oct;62(10):1608–28.

14. Zhao XF, Wan J, Powell C, Ramachandran R, Myers MG Jr, Goldman D. Leptin and IL-6 family cytokines synergize to stimulate muller glia reprogramming and retina regeneration. Cell Rep. 2014 Oct 9;9(1):272–84.

15. Wan J, Ramachandran R, Goldman D. HB-EGF is necessary and sufficient for Muller glia dedifferentiation and retina regeneration. Dev Cell. 2012 Feb 14;22(2):334–47.

16. Ramachandran R, Zhao XF, Goldman D. Ascl1a/Dkk/beta-catenin signaling pathway is necessary and glycogen synthase kinase-3beta inhibition is sufficient for zebrafish retina regeneration. Proc Natl Acad Sci U A. 2011 Sep 20;108(38):15858–63.

17. Conner C, Ackerman KM, Lahne M, Hobgood JS, Hyde DR. Repressing Notch Signaling and Expressing TNFalpha Are Sufficient to Mimic Retinal Regeneration by Inducing Muller Glial Proliferation to Generate Committed Progenitor Cells. J Neurosci. 2014 Oct 22;34(43):14403–19.

18. Nelson CM, Ackerman KM, O'Hayer P, Bailey TJ, Gorsuch RA, Hyde DR. Tumor necrosis factor-alpha is produced by dying retinal neurons and is required for Muller glia proliferation during zebrafish retinal regeneration. J Neurosci. 2013 Apr 10;33(15):6524–39.

19. Jorstad NL, Wilken MS, Grimes WN, Wohl SG, VandenBosch LS, Yoshimatsu T, et al. Stimulation of functional neuronal regeneration from Muller glia in adult mice. Nature [Internet]. 2017 Jul 26; Available from: https://www.ncbi.nlm.nih.gov/pubmed/28746305

20. Pollak J, Wilken MS, Ueki Y, Cox KE, Sullivan JM, Taylor RJ, et al. ASCL1 reprograms mouse Muller glia into neurogenic retinal progenitors. Development. 2013 Jun;140(12):2619–31.

21. Ueki Y, Wilken MS, Cox KE, Chipman L, Jorstad N, Sternhagen K, et al. Transgenic expression of the proneural transcription factor Ascl1 in Muller glia stimulates retinal regeneration in young mice. Proc Natl Acad Sci U A. 2015 Nov 3;112(44):13717–22.

22. Yao K, Qiu S, Wang YV, Park SJH, Mohns EJ, Mehta B, et al. Restoration of vision after de novo genesis of rod photoreceptors in mammalian retinas. Nature. 2018 Aug;560(7719):484–8.

23. Fausett BV, Goldman D. A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. J Neurosci. 2006 Jun 7;26(23):6303–13.

24. Moritz LE, Trievel RC. Structure, mechanism, and regulation of polycomb repressive complex 2. J Biol Chem [Internet]. 2017 Sep 14; Available from: https://www.ncbi.nlm.nih.gov/pubmed/28912274

25. Shen C, Vakoc CR. Gain-of-function mutation of chromatin regulators as a tumorigenic mechanism and an opportunity for therapeutic intervention. Curr Opin Oncol. 2015 Jan;27(1):57–63.

26. Ueno K, Iwagawa T, Ochiai G, Koso H, Nakauchi H, Nagasaki M, et al. Analysis of Muller glia specific genes and their histone modification using Hes1-promoter driven EGFP expressing mouse. Sci Rep. 2017 Jun 15;7(1):3578.

27. Aldiri I, Moore KB, Hutcheson DA, Zhang J, Vetter ML. Polycomb repressive complex PRC2 regulates Xenopus retina development downstream of Wnt/beta-catenin signaling. Development. 2013 Jul;140(14):2867–78.

28. Zhang J, Taylor RJ, La Torre A, Wilken MS, Cox KE, Reh TA, et al. Ezh2 maintains retinal progenitor proliferation, transcriptional integrity, and the timing of late differentiation. Dev Biol. 2015 Jul 15;403(2):128–38.

29. Yan N, Cheng L, Cho K, Malik MT, Xiao L, Guo C, et al. Postnatal onset of retinal degeneration by loss of embryonic Ezh2 repression of Six1. Sci Rep. 2016 Sep 28;6:33887.

30. Rapicavoli NA, Poth EM, Zhu H, Blackshaw S. The long noncoding RNA Six3OS acts in trans to regulate retinal development by modulating Six3 activity. Neural Dev. 2011 Sep 21;6:32.

31. Hoang T, Wang J, Boyd P, Wang F, Santiago C, Jiang L, et al. Cross-species transcriptomic and epigenomic analysis reveals key regulators of injury response and neuronal regeneration in vertebrate retinas. bioRxiv. 2019 Jul 31;717876.

32. Gallina D, Todd L, Fischer AJ. A comparative analysis of Muller glia-mediated regeneration in the vertebrate retina. Exp Eye Res. 2014 Jul 9;123:121–30.

33. Fischer AJ, Bongini R. Turning Muller glia into neural progenitors in the retina. Mol Neurobiol. 2010 Dec;42(3):199–209.

34. Landis SC, Amara SG, Asadullah K, Austin CP, Blumenstein R, Bradley EW, et al. A call for transparent reporting to optimize the predictive value of preclinical research. Nature. 2012 Oct 11;490(7419):187–91.

35. Steward O, Balice-Gordon R. Rigor or mortis: best practices for preclinical research in neuroscience. Neuron. 2014 Nov 5;84(3):572–81.

36. Fischer AJ, Scott MA, Tuten W. Mitogen-activated protein kinase-signaling stimulates Muller glia to proliferate in acutely damaged chicken retina. Glia. 2009 Jan 15;57(2):166–81.

37. Ghai K, Zelinka C, Fischer AJ. Notch signaling influences neuroprotective and proliferative properties of mature Muller glia. J Neurosci. 2010 Feb 24;30(8):3101–12.

38. Zelinka CP, Volkov L, Goodman ZA, Todd L, Palazzo I, Bishop WA, et al. mTor signaling is required for the formation of proliferating Muller glia-derived progenitor cells in the chick retina. Development. 2016 Jun;143(11):1859–73.

39. Todd L, Volkov LI, Zelinka C, Squires N, Fischer AJ. Heparin-binding EGF-like growth factor (HB-EGF) stimulates the proliferation of Muller glia-derived progenitor cells in avian and murine retinas. Mol Cell Neurosci. 2015 Nov;69:54–64.

40. Todd L, Suarez L, Quinn C, Fischer AJ. Retinoic Acid-Signaling Regulates the Proliferative and Neurogenic Capacity of Muller Glia-Derived Progenitor Cells in the Avian Retina. Stem Cells [Internet]. 2018 Nov 27;36(392–405). Available from: https://www.ncbi.nlm.nih.gov/pubmed/29193451

41. Todd L, Squires N, Suarez L, Fischer AJ. Jak/Stat signaling regulates the proliferation and neurogenic potential of Muller glia-derived progenitor cells in the avian retina. Sci Rep. 2016 Oct 19;6:35703.

42. Todd L, Fischer AJ. Hedgehog-signaling stimulates the formation of proliferating Müller glia-derived progenitor cells in the retina. Development. 2015;142:2610–22.

43. Todd L, Palazzo I, Squires N, Mendonca N, Fischer AJ. BMP- and TGFbeta-signaling regulate the formation of Muller glia-derived progenitor cells in the avian retina. Glia [Internet]. 2017 Jul 13; Available from: https://www.ncbi.nlm.nih.gov/pubmed/28703293

44. Prada C, Puga J, Perez-Mendez L, Lopez R, Ramirez G. Spatial and Temporal Patterns of Neurogenesis in the Chick Retina. Eur J Neurosci. 1991 Jun;3(6):559–69.

45. Fischer AJ, Zelinka C, Gallina D, Scott MA, Todd L. Reactive microglia and macrophage facilitate the formation of Muller glia-derived retinal progenitors. Glia. 2014 Oct;62(10):1608–28.

46. Gallina D, Palazzo I, Steffenson L, Todd L, Fischer AJ. Wnt/betacatenin-signaling and the formation of Muller glia-derived progenitors in the chick retina. Dev Neurobiol [Internet]. 2015 Dec 9; Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=26663639

47. Gallina D, Zelinka C, Fischer AJ. Glucocorticoid receptors in the retina, Muller glia and the formation of Muller glia-derived progenitors. Development. 2014 Sep;141(17):3340–51.

48. Palazzo I, Deistler K, Hoang TV, Blackshaw S, Fischer AJ. NF-κB signaling regulates the formation of proliferating Müller glia-derived progenitor cells in the avian retina. bioRxiv. 2019 Aug 2;724260.

49. Pan YM, Wang CG, Zhu M, Xing R, Cui JT, Li WM, et al. STAT3 signaling drives EZH2 transcriptional activation and mediates poor prognosis in gastric cancer. Mol Cancer. 2016 Dec 9;15(1):79.

50. Fischer AJ, Scott MA, Ritchey ER, Sherwood P. Mitogen-activated protein kinase-signaling regulates the ability of Müller glia to proliferate and protect retinal neurons against excitotoxicity. Glia. 2009;57(14):1538–52.

51. Fischer AJ, Zelinka C, Milani-Nejad N. Reactive retinal microglia, neuronal survival, and the formation of retinal folds and detachments. Glia. 2015 Feb;63(2):313–27.

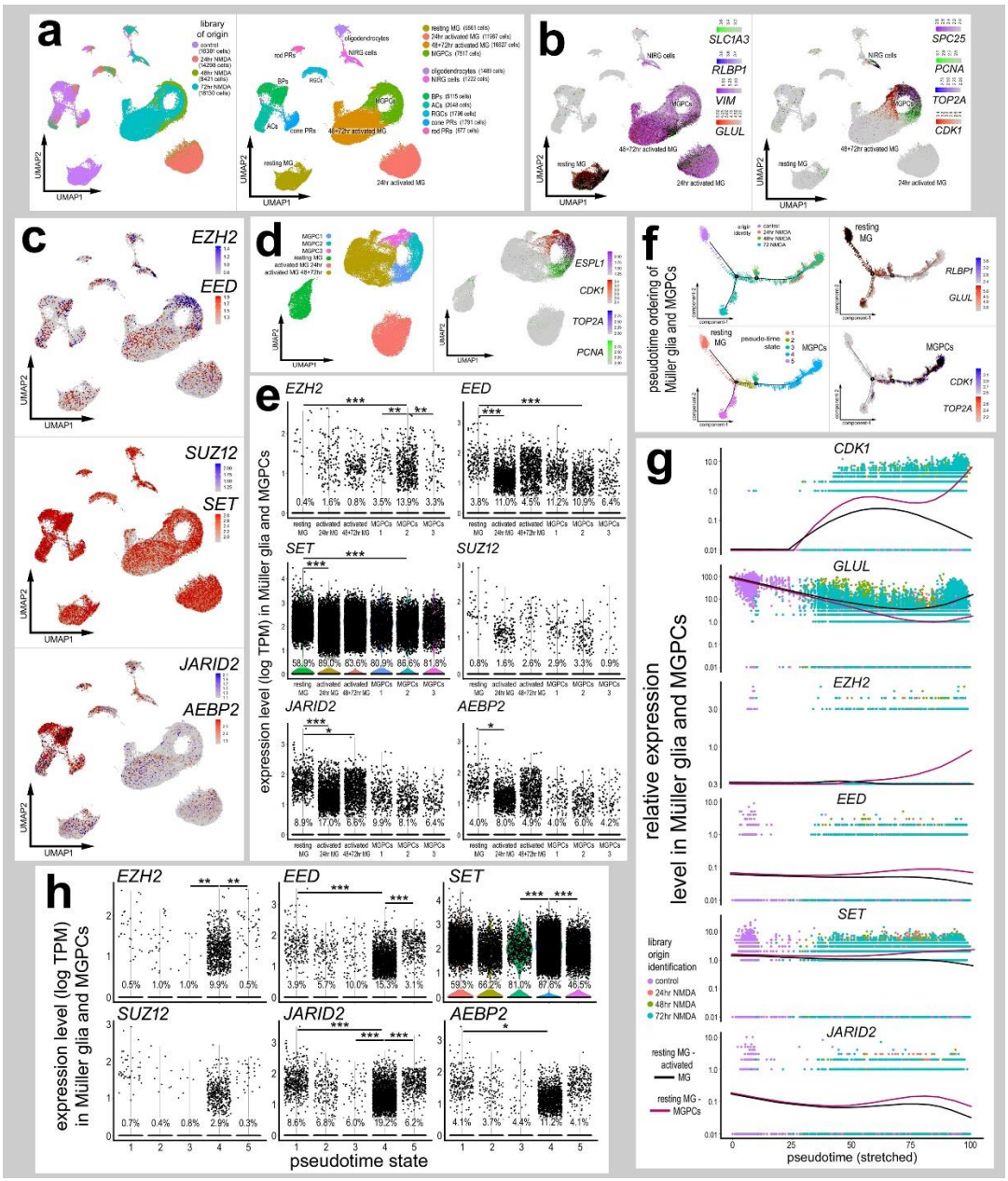

Figure 1. Expression of PRC2-related(Ghai et al., 2010; Palazzo et al., 2019; Todd and Fischer, 2015; Todd et al., 2016; Zelinka et al., 2016) genes in retinal cells following NMDA-treatment of the chick retina. scRNA-seq was used to identify patterns of expression of EZH2-related genes among acutely dissociated retinal cells. In tSNE (**a**-**c**), violin (**d**,**g**,**j**), pseudotime (**e**,**f**) and UMAP plots (**h**,**i**), each dot represents one cell. In tSNE and UMAP plots black dots demonstrate individual cells that express 2 or more markers. Cells were obtained from control retinas (16,595

cells), and from retinas at 24hrs (14,918 cells), 48hrs (8483 cells) and 72 hrs (18,338 cells) after NMDA-treatment (**a**). tSNE plots revealed distinct clustering of different types of retinal cells; control MG (5468 cells), 24 hr NMDA-treated MG (12,576 cells), 48+72 hrs NMDA-treated MG (18,848 cells), MGPCs (5336 cells), microglia (66 cells), oligodendrocytes (1494 cells), NIRG cells (1224 cells), retinal ganglion cells (RGCs; 2049 cells), amacrine cells (2635 cells), bipolar cells (5169 cells), rod photoreceptors (974 cells), and cone photoreceptors (2495 cells) Pseudotime trajectories for MG and MGPCs revealed a branched trajectory with resting MG, proliferating MGPCs, and activated MG from 72hr after NMDA treatment largely confined to different branches (**e**). UMAP plots illustrate distinct clustering of MG from 48hrs after NMDA treated plus/minus insulin+FGF2 treatment (**h**). UMAP plots demonstrate patterns of expression for GLUL, RLBP1 and *VIM* (Muller glia), *TOP2A*, *PCNA* and *CDK1* (MGPCs), (**h**, **i**). Violin/scatter plots indicate significant differences (**p<0.001, ***p<<0.0001; Wilcox rank sum with Bonferroni correction) in expression of *GLUL*, *CDK1*, TOP2A among MG and MGPCs (**j**).

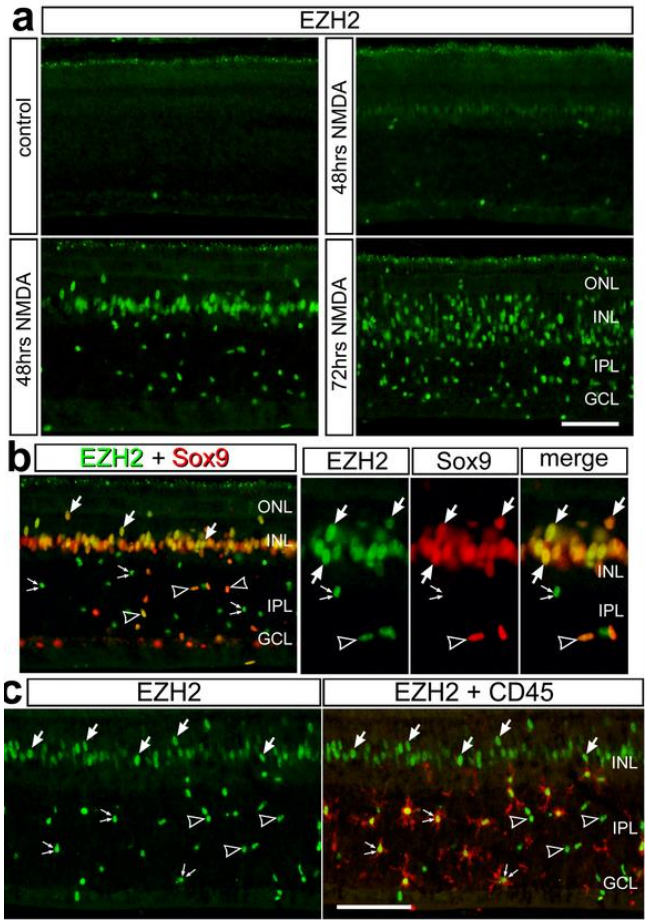

Figure 3. EZH2 immunolabelling increased in proliferating MGPCs in damaged retinas.

Chick eyes were injected with NMDA. Eyes were harvested at 24 hrs after the last injection and retinas processed for immunolabeling. Retinas were labeled for Sox2 (red) and $EZH2^{+}$ (green) cells (**a**,**b**), or CD45 (**c**). Arrows indicate the nuclei of MG. The calibration bar panels **a, b** and **c** represent 50 µm. Abbreviations: ONL – outer nuclear layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer, NF – neurofilament

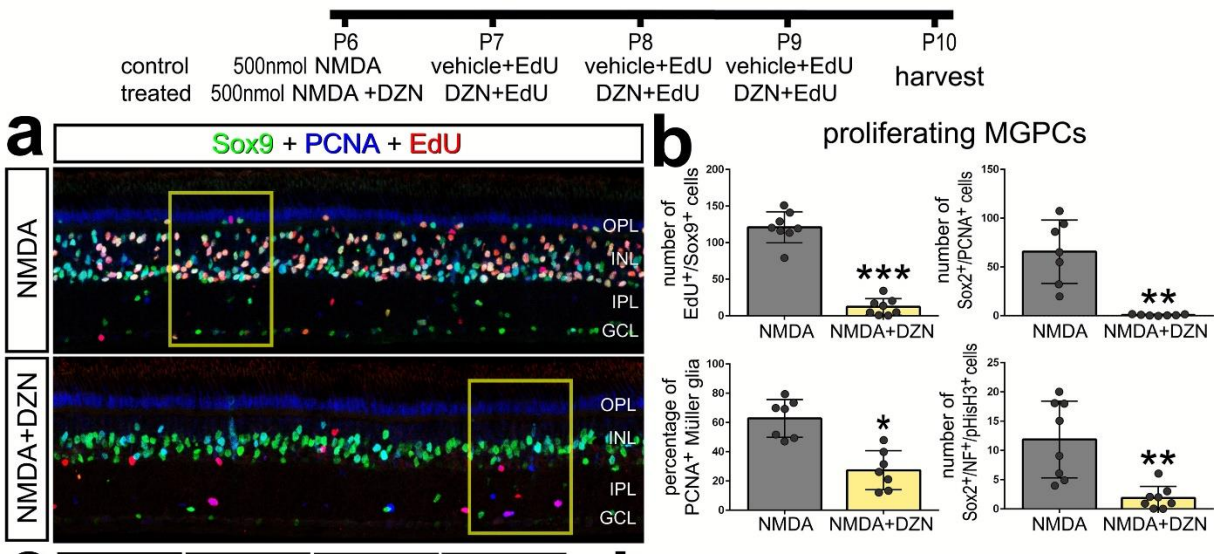

Figure 3. DZN decreases numbers of proliferating MGPCs in damaged retinas.

Chick eyes were injected with NMDA, DZN, and EdU according to the paradigm at the top figure. Eyes were harvested at 24 hrs after the last injection and retinas processed for immunolabeling. Retinas were labeled for Sox9 (green) and EdU^+ (red) cells (**a**), and PCNA (blue). Histograms illustrate the mean (± SD) and each dot represents one biological replicate. Significance of difference (**p<0.01, ***p<0.001) was determined by using a paired *t*-test. Arrows indicate the nuclei of MG. The calibration bar panel **a** represent 50 µm. Abbreviations: ONL – outer nuclear layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer, NF – neurofilament,

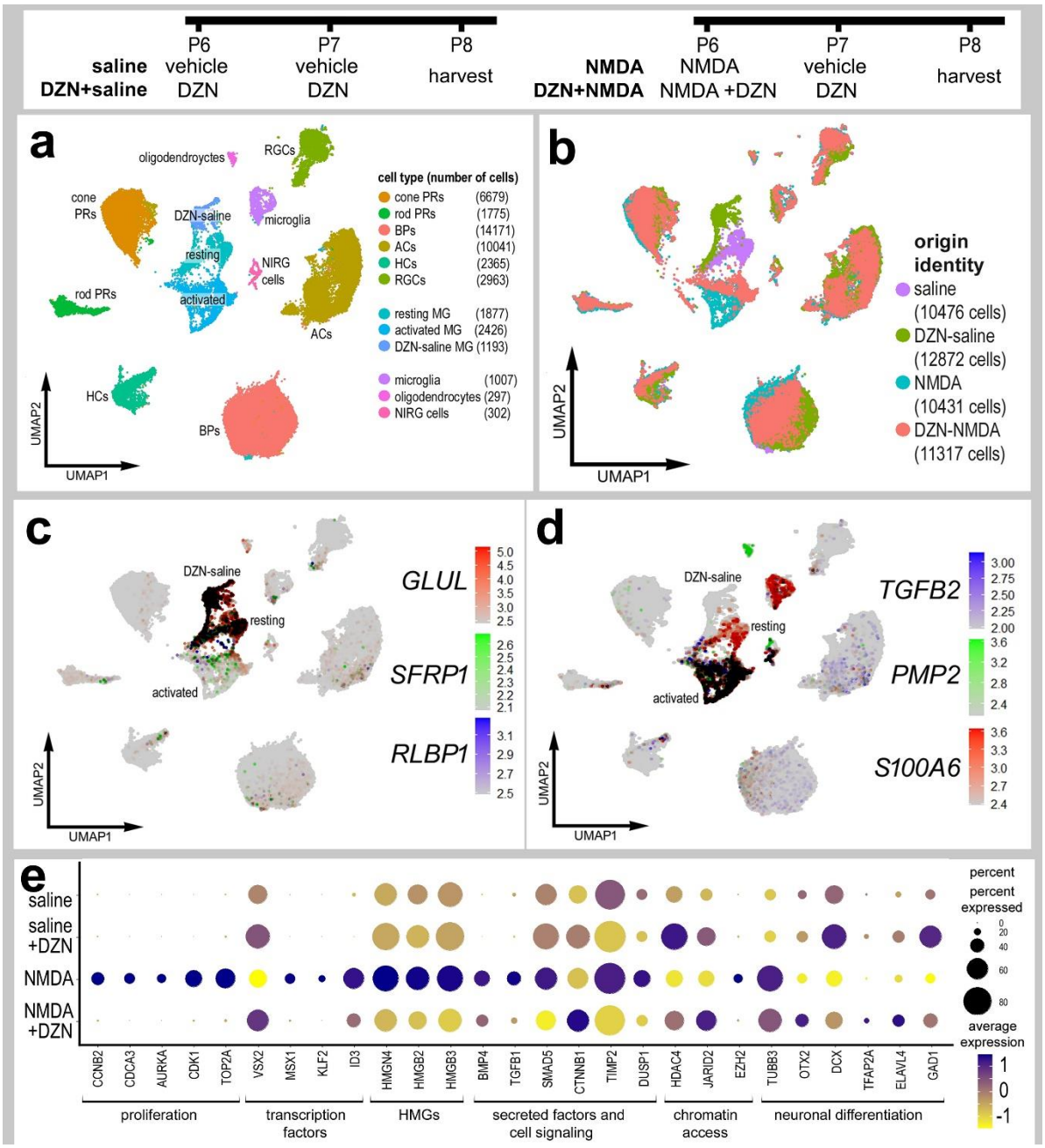

Figure 4. Inhibition of EZH2 with DZN results in a significant downregulation of proliferation genes and genes for neuronal differentiation. genes in retinal cells following NMDA-treatment of the chick retina. scRNA-seq was used to identify patterns of expression of proliferation and neurodifferentiation-related genes among acutely dissociated retinal cells. In UMAP (**a-c**), violin each dot represents one cell. UMAP plots demonstrate patterns of clustering for differently

treated retina for Müller glia. The dot plot is organized by proliferation, transcription factor, HMG, secreted factor, chromatin access, and neuronal differentiation among the different treatments. They are colorized by feature changes to highlight the changes between the different features listed in the plot.